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Novel Insertion Mutation in a Non-Jewish Caucasian Type 1 Gaucher Disease Patient

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Gaucher disease is the most prevalent lysosomal storage disorder. It is autosomalrecessive, resulting in lysosomal glucocerebrosidase deficiency. Three clinical forms of Gaucher disease have been described: type 1 (nonneuronopathic), type 2 (acute neuronopathic), and type 3 (subacute neuronopathic). We performed PCR-thermal cycle sequence analysis of glucocerebrosidase genomic DNA and identified a novel mutation in a non-Jewish type 1 Gaucher disease patient. It is a C insertion in exon 3 at cDNA nucleotide position 122 and genomic nucleotide position 1626. This mutation causes a frameshift and, subsequently, four of the five codons immediately downstream of the insertion were changed while the sixth was converted to a stop codon, resulting in premature termination of protein translation. The 122CC insertion abolishes a *Cac*81 restriction endonuclease cleavage site, allowing a convenient and reliable method for detection using RFLP analysis of PCR- amplified glucocerebrosidase genomic DNA. The mutation in the other Gaucher allele was found to be an A→G substitution at glucocerebrosidase cDNA nucleotide position 1226 that so far has only been reported among type 1 Gaucher disease patients. Since mutation 122CC causes a frameshift and early termination of protein translation, it most likely results in a meaningless transcript and subsequently no residual glucocerebrosidase enzyme activity. We speculate that mutation 122CC may result in a worse prognosis than mutations associated with partial activity. When present in the homozygous form, it could be a lethal allele similar to what has been postulated for the other known insertion mutation, 84GG. Our patient, who is a compound heterozygote 122CC/1226G, has moderately severe type 1 Gaucher disease. Her clinical response to *Ceredase*® therapy that began 31 months ago has been favorable, though incomplete. Am. J. Med. Genet. 68:211–215, 1997 © 1997 Wiley-Liss, Inc.

KEY WORDS: Gaucher disease; glucocerebrosidase; ceredase therapy; mutation; PCR

INTRODUCTION

Gaucher disease is an inherited sphingolipidosis resulting from deficient activity of the lysosomal enzyme glucocerebrosidase (EC.3.2.1.45) [Brady et al., 1965; Patrick, 1965]. It is the most prevalent lysosomal storage disease, transmitted as an autosomal-recessive trait [Beutler and Grabowski, 1995; Brady and Barranger, 1983]. Three main clinical forms of Gaucher disease have been described: type 1, nonneuronopathic; type 2, acute neuronopathic; and type 3, subacute neuronopathic. Although Gaucher disease is panethnic, it is more common in Ashkenazi Jews, with a gene frequency estimated at 0.035–0.040 [Beutler, 1992; Zimran et al., 1991]. Four public mutations constitute >96% of the total mutations among Jewish patients [Beutler et al., 1993]. These are: an A→G transition at glucocerebrosidase cDNA nucleotide position (nt) 1226 that results in ^{370}Asn→^{370}Ser [Tsuji et al., 1988; Theophilus et al., 1989]; a G insertion at nt 84 [Beutler et al., 1991]; a T→C transition at nt 1448 that results in ^{444}Leu→^{444}Pro [Tsuji et al., 1987; Wigderson et al., 1989]; and a splicing mutation in intron 2 (g→a) [Beutler et al., 1992; He and Grabowski, 1992]. Among non-Jewish patients, mutations 1448C and 1226G constitute >50% of the total, while approximately one fourth to one third of mutations remain unidentified [Beutler, 1992]. In this report, we describe the identification of a novel insertion mutation in a non-Jewish type 1 Gaucher disease patient, and a simple diagnostic method that utilizes PCR amplification of glucocerebrosidase genomic DNA and restriction endonuclease analysis for its identification. In addition, the clinical effects of the mutation, and results of replacement enzyme therapy with *Ceredase*®, are discussed.

CLINICAL REPORT

Patient L.B. is a non-Jewish Caucasian female who has 4 unaffected sib. Her parents are nonconsan-

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guineous and are both originally of Russian descent. She presented at age 5 years with hepatosplenomegaly detected on a routine physical examination. The liver was 5 cm, and the spleen 6 cm, below the costal margin. Height was 106 cm (10th centile), and weight was 17.1 kg (3rd centile). Hemoglobin was 98 g/l, white blood cell (WBC) 5,000/cubic mm, platelets 87,000/cubic mm. A bone-marrow examination showed Gaucher cells. β-glucosidase activity determined from cultured fibroblasts by our assay procedure [Choy and Davidson, 1980] was 5.5 nmol/hr/mg protein (13.8% of mean normal control values). Throughout childhood she had slowly increasing organomegaly and abdominal distension, with moderate anemia. Growth was mildly delayed, but adult stature was normal (163 cm). There was no skeletal symptomatology, though X-ray studies demonstrated "Erlenmeyer flask" deformities of her distal femura. At age 19 years, she was reevaluated because of increasing abdominal discomfort and dyspnea. There was evidence of multiple splenic infarcts and gastroesophageal reflux. Consideration was given to bone-marrow transplantation, and one of her sibs was HLA-identical. However, she chose not to undergo the procedure at that time. *Ceredase*® therapy was started at age 24, and the clinical response was favorable, though incomplete, as demonstrated in Table I. Clinical severity, using the scoring system of Zimran et al. [1992], was estimated to be 5 at diagnosis (age 5), 11 at start of *Ceredase*® therapy, and reduced to 5 most recently. Problems encountered during therapy have included marked weight gain, depression, hypertension, and proteinuria. The reason for the rapid gain in body weight (23 kg in about 2 years) is not entirely clear, but is most likely related to concurrent depression and possibly increased appetite with decreasing organomegaly, while the hypertension may be primary and related to her obesity or the renal lesion.

MATERIALS AND METHODS

Fibroblasts were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum [Choy et al., 1994]. Genomic DNA was isolated from fibroblasts using the DNAZOL™ kit (GIBCO-BRL, Gaithersburg, MD).

A two-stage nested PCR amplification method was used for selective amplification of the glucocerebrosidase functional gene, but not the pseudogene which shares >96% sequence homology with the functional gene [Horowitz et al., 1989; Zimran et al., 1990]. In the first PCR amplification, primers with nucleotide sequence deleted in the pseudogene were used to selectively amplify the glucocerebrosidase functional gene from genomic DNA. As shown in Table II, glucocerebrosidase exons 1–6 are flanked by primers A and B, while glucocerebrosidase exons 7–11 are flanked by primers C and D. Primer B is antisense to glucocerebrosidase genomic nt 3813–3793, and primer C is sense to glucocerebrosidase genomic nt 3831–3850. These nucleotide sequences are deleted in the pseudogene [Horowitz et al., 1989]. Therefore, these primers will flank and amplify the glucocerebrosidase functional gene but not the pseudogene in the first PCR. Gluco-

TABLE I. Clinical Progress and Results of Treatment*

Age (years)	Weight (kg)	Hemoglobin (g/l) (115–160)	Platelet ($\times 10^9$/L) (140–340)	Retics (ab) (20–85 $\times 10^9$/L)	ESR (0–15)	Acid phos. (IU/l) (<0.8)	ALT (IU/l) (0–30)	Alk. phos. (IU/l) (40–130)	Ceredase dose (units)		Clinical assessment	
									Freq./wk	U/kg/mo	Liver (cm) (below costal margin)	Spleen (cm)
5.5	17.1	98	87		ND	1.9	98	224			5	6
18.9	ND	81	127	278	ND	ND	99	191			4	"iliac crest"
24.3	53.6	106	105	ND	ND	ND	ND	185	3	67	20	17
24.6	59.1	106	88	150	46	1.3	ND	199	3	61	20	20
24.9	63.6	117	73	191	43	1.1	ND	141	3	28	14	15
25.2	66.4	116	128	210	32	0.9	ND	138	1	27	15	14
26.1	72.0	129	185	195	42	0.7	66	152	2	28	11	12
26.3	74.2	132	215	189	35	0.4	72	123	3	40	10	10
26.8	76.8	120	197	141	33	3.0[a]	77	117	2	39	10	9
27.0	75.4	132	214	139	26	2.9[a]	106	125	2	40	9	8

*Retics, reticulocytes count; ESR, erythrocyte sedimentation rate; Acid. phos., acid phosphatase; ALT, alanine transaminase; Alk. phos., alkaline phosphatase; Freq/wk, frequency per week; U/Kg/Mo, units per kilogram body weight per month; ND, not done. The numbers in parentheses indicate normal values.
[a]The methodology in acid phosphatase activity assay was changed during the patient's last two visits which may partially explain the increase in acid phosphatase activity in the patient (normal values for the new method were <1.2 IU/L).

TABLE II. Primers for PCR Amplification of Glucocerebrosidase Genomic DNA

Primers	Nucleotide sequence (5′ → 3′)	Orientation and location
A	CGGAATTACTTGCAGGGCTA	Sense to genomic nt 446–465[a] cDNA nt minus 137–118[b]
B	TGGGTGACAGAGAGAGAGACT	Antisense to genomic nt 3813–3793 deleted in the pseudogene
C	GCCATCTTCACTCACTGTAA	Sense to genomic nt 3831–3850 deleted in the pseudogene
D	CTTTAATGCCCAGGCTGAGC	Antisense to genomic nt 6737–6718 downstream of TGA stop codon at genomic nt 6688–6690
E	CTCGGCCTCCTAAAGTGCTA	Sense to genomic nt 1464–1483
F	AAGGATGTTGAGAGCAGCA	Antisense to genomic nt 2026–2008

[a]Genomic nucleotide position is numbered according to Horowitz et al. [1989].
[b]cDNA nucleotide position is numbered according to Sorge et al. [1987], where the A of the first upstream ATG initiation site of glucocerebrosidase gene is position no. 1.

cerebrosidase genomic DNA amplified by this PCR was then used as template for the amplification of each of the individual exons. In the second PCR amplification, each of the glucocerebrosidase exons was amplified by using a pair of primers that flank the intron regions approximately 15 bp upstream and downstream of that exon. This covers the entire coding region of the glucocerebrosidase gene, as well as potential sites in introns for splicing mutations. PCR amplifications were performed as previously described [Choy et al., 1994], with one minor modification: denaturation of genomic DNA in the first step of the first cycle was extended to 10 min at 94°C to ensure complete separation of the two strands. Taq polymerase was then added when the temperature was lowered to 58°C for primer annealing and subsequent elongation at 72°C.

DNA sequence analysis was performed using the dideoxynucleotide chain termination method by Sanger et al. [1977] and the FMOL™ DNA Sequencing System (Promega Corporation, Madison, WI), as described previously [Choy et al., 1994]. The PCR mismatch method [Beutler et al., 1990] and *Xho*I restriction endonuclease analysis were used for the detection of mutation 1226G

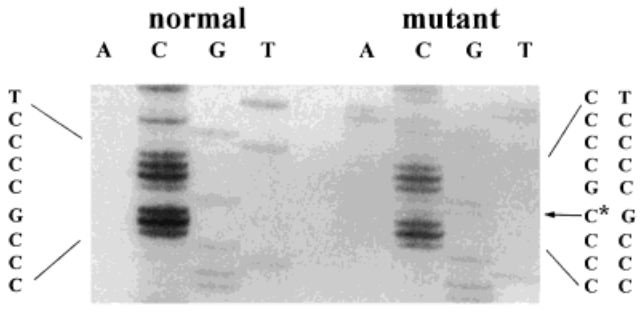

Fig. 1. DNA sequence analysis of PCR-amplified genomic DNA of glucocerebrosidase exon 3 using the chain termination method of Sanger et al. [1977]. The A, C, G, and T lanes at left are control, and lanes at right are the patient. Note that direct sequencing was performed on the PCR-amplified glucocerebrosidase genomic DNA of both alleles. Subsequently, when a C insertion mutation was identified (marked by an asterisk) in one of the alleles of the patient, the insertion and all of the sequence 3′ to the insertion are not in frame with the nucleotide sequence in the second allele of the patient. This results in the appearance of double bands.

[Choy et al., 1994, 1996]. For mutation 122CC, glucocerebrosidase exon 3 amplified from genomic DNA by the PCR method using primers E and F (Table II) was digested with *Cac*81 restriction endonuclease and analyzed by agarose gel electrophoresis.

RESULTS

The results of DNA sequence analysis of glucocerebrosidase exon 3 from the control and from patient L.B. are shown in Figure 1. It was noted that an additional C at cDNA nt 122 (genomic nt 1626) was present in the heterozygous form in the patient. This insertion resulted in a frameshift mutation in one allele, while the second allele sequence remained normal. Since direct sequence analysis was performed on PCR-amplified genomic DNA from both alleles, the insertion created a frameshift which resulted in double bands at, and downstream of, the insertion site (Fig. 1).

Since the 122CC insertion mutation created a frameshift, the codons downstream of the mutation were simultaneously shifted and altered. A computer analysis of the normal and mutated sequence is shown in Figure 2. As shown, four out of the five codons immediately 3′ to the insertion were changed, while the sixth was converted to a stop (TAA) codon. This results in early termination of protein translation for the glucocerebrosidase polypeptide and subsequently the synthesis of a truncated and most likely nonfunctional enzyme.

A computer search for a restriction cleavage site altered by the mutation showed that a *Cac*81 cleavage site would be abolished. Subsequently, *Cac*81 restriction endonuclease analysis was used to detect and confirm the presence of this mutation. Glucocerebrosidase intron 2 and exon 3 of the functional gene, and not the pseudogene, were amplified from genomic DNA of a normal control and from patient L.B. by the PCR method using specific primers E (deleted in the pseudogene) and F (Table II), digested by *Cac*81 restriction endonuclease, and analyzed by agarose gel electrophoresis. As shown in Figure 3, there were two *Cac*81 sites in the control which generated three DNA fragments of 313, 157, and 93 bp. In patient L.B., the 122CC insertion abolished the first cleavage site, resulting in only two digested fragments of 470 and 93 bp. As noted in

Normal nucleotide sequence	5'.....GCC CGC CCC TGC ATC CCT AAA AGC..... 3'
Amino acids encoded	.....Ala Arg Pro Cys Ile Pro Lys Ser.....
Mutant nucleotide sequence	5'.....GCC CC*G CCC CTG CAT CCC TAA AAG..... 3'
Amino acid encoded	.....Ala Pro Pro Leu His Pro **STOP**

Fig. 2. Frameshift and alterations created by insertion mutation 122CC. The codon sequence of glucocerebrosidase cDNA nucleotide position 118–141 and the respective amino acids encoded from the control and the patient are shown. Asterisk at nucleotide position 122 of the patient is the site of the insertion mutation. Note that four of the five amino acids encoded are altered, while the codon for the sixth amino acid (AAA for lysine) was changed to TAA which is a stop codon, resulting in premature termination of glucocerebrosidase protein translation.

lane 2 of Figure 3, an undigested 470-bp band from the allele with the insertion as well as the digested 313- and 157-bp bands from the other allele were present, thus confirming the presence of mutation 122CC in heterozygous form. This finding also eliminates the possibility that the results from sequence analysis were due to Taq polymerase incorporation error.

The mutation in the other allele was also identified by sequence analysis. It was a A → G transition mutation at cDNA nt 1226 that resulted in ^{370}Asn→^{370}Ser of glucocerebrosidase, present in heterozygous form. The presence of this heterozygous mutation in patient L.B.

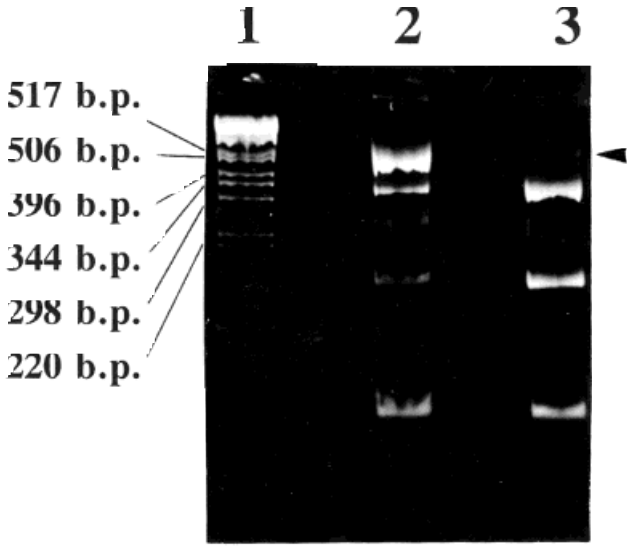

Fig. 3. *Cac*81 restriction endonuclease analysis of mutation 122CC. Conditions of restriction enzyme digestion and agarose gel electrophoresis were described previously [Choy et al., 1994]. **Lane 1**, DNA standards (1-kb ladder from *Hinf*I-digested DNA fragments (New England BioLabs) of 517, 506, 396, 344, 298, 220, and 201 bp; **lane 2**, PCR-amplified genomic DNA of glucocerebrosidase exon 3 of Gaucher disease patient L.B. after digestion; and **lane 3**, similar sample from a normal control. In the control, there are two *Cac*81 cleavage sites that result in three digested bands of 317, 157, and 93 bp (lane 3). The mutation in the patient abolishes a restriction cleavage site, resulting in an undigested DNA band of 470 bp (lane 2, first band from top as indicated by arrowhead) and a digested band of 157 bp. Since the mutation is present in heterozygous form, digested bands of 317 and 93 bp from the other allele are also present.

was confirmed by the mismatched PCR method and *XhoI* restriction analysis [Choy et al., 1994], adapted from Beutler et al. [1990] (data not shown). DNA sequence analysis of the rest of the glucocerebrosidase coding region and intron-exon boundaries showed identity with normal controls.

DISCUSSION

Since Tsuji et al. [1987] identified the first mutation (1448C) in Gaucher disease, three other public mutations and more than 35 private mutations have been reported [reviewed in Beutler et al., 1994]. The majority of these are missense mutations that result in amino-acid substitution in the glucocerebrosidase polypeptide [Beutler et al., 1994]. The 122C mutation described in this paper is the second insertion type so far reported, the first being the 84GG mutation [Beutler et al., 1991]. Since the insertion occurs at the beginning of the third of 11 exons of the glucocerebrosidase functional gene, a frameshift and a subsequent early stop codon are created (Fig. 2). This would likely render the messenger transcript meaningless and the encoded gene product nonfunctional. Miao et al. [1994] reported that the catalytic nucleophile in the active site of the enzyme was at Glu^{340} encoded by exon 8 of the glucocerebrosidase functional gene. Thus, it is unlikely that a truncated glucocerebrosidase, without the active site, will possess any residual activity. In this regard, mutation 122CC may resemble the null allele or "knockout" mutation in the mouse [Tybulewicz et al., 1992] and human [Sidransky et al., 1992; Strasberg et al., 1993]. A similar idea was also postulated for the 84GG insertion mutation [Beutler, 1992], where it was suggested that in the homozygous form, there would probably be a completely nonfunctional glucocerebrosidase. A complete deficiency of glucocerebrosidase activity is likely incompatible with life and results in either spontaneous abortion or death shortly after birth. This idea is supported by the observation that there has been no patient with Gaucher disease homozygous for the 84GG insertion mutation, despite the fact that the carrier frequency of this mutation is the second highest (13%) in Jewish populations. Furthermore, the clinical course of Gaucher disease in patients who are homozygous for

mutation 1226 is generally milder than those with the 84G/1226 genotype [Sibille et al., 1993; Zimran et al., 1989]. The clinical severity of patient L.B., who is a compound heterozygote 122CC/1226G, could be described as moderately severe type 1 Gaucher disease, with a severity score of 11 at onset of *Ceredase*® treatment. The replacement enzyme has been mostly used at lower dose levels, and clinical response has been favorable, though incomplete.

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